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QUANTITATIVE VIROLOGIC STUDIES OF SEWAGE

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COUNTY OF SAN DIEGO

January 25, 1968

TO: J. B. Askew, M.D.
Director of Public Health

FROM: Raymond E. Leach, Chief
Public Health Laboratory

SUBJECT: SECOND PROGRESS REPORT - RESEARCH GRANT NO. 1WP-01198-01
QUANTITATIVE VIROLOGIC STUDIES OF SEWAGE
For Transmission to the Division of Grants Management - WPCA

I. Administrative Aspects

A. Dates of this Report

The period of this report is the regular calendar quarter October through December 1967.

B. Personnel

Efforts by the County Personnel Section and the staff of this laboratory are continuing toward recruitment of qualified individuals to fill two microbiologist openings in the virus section, one for assisting with this grant and another for the diagnostic activities of this section. An experienced laboratory assistant was transferred to the virus section on December 1, to a position authorized by this grant and her position in the main laboratory was re-filled by the employment of a new laboratory assistant. This is in addition to routine services rendered to the viral section by the laboratory aid or assistants assigned to glassware preparation.

C. Equipment

The 25ml cup attachment, which had been ordered for the Biosonik II sonifier as a part of the capital outlay authorized for this project, was received October 23. This will permit sonication of virus suspensions in closed vials and inserted into the water in the cup attachment. A roller drum ordered September 26 for this project was received on December 11, but the rotator for which this drum was to be used, ordered at the same time, still had not been received at the time of preparation of this report.

A wall cabinet, ordered on the regular county budget to make more table space available for work in the virus section, was received in December but the company had sent the wrong item and it had to be returned for exchange.

This concludes the Administrative Aspects of this report. The technical report which follows has been prepared by the principal investigator, Mrs. Beatrice England.

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II. Statement of the Problem

Quantitation of the major virus groups present in sewage involves meeting the following requirements:

A. Selection of an applicable method of assay.

B. Concentration of viral content.

C. Separation of viral mixtures, so that measurement of one group does not preclude recognition of other virus groups.

D. A sensitive host system for each virus group that is to be cultured.

E. Identification of the virus groups.

To date, experiments have been directed toward the first two of these requirements, and results of preliminary tests are presented herein.

III. Experimental Aspects

A. Summary of work performed during this quarterly period

Preparatory to virus concentration experiments, stock laboratory strains of enterovirus, adenovirus, and reovirus were titrated by one or more of the following methods: enumeration of plaque forming units (PFU), determination of most probable number (MPN), or determination of the 50% tissue culture infective dose(TCD_{50}) per unit volume. This permitted preliminary comparative studies of these three assay systems, including modifications such as use of pancreatin in plaque overlay medium for reovirus (1).

Experiments were performed on concentration of viruses by adsorption to calcium and aluminum precipitates, as described by Wallis and Melnick (2). These precipitates were found to be efficient adsorbents for viruses. Studies were initiated on the effect of certain technical variables, such as presence of serum in the virus-precipitate mixture, and washing of the precipitate following the adsorption period. All of these investigations are continuing.

B. Viruses employed

Representatives were selected from the three major virus groups commonly recovered from sewage: enterovirus, adenovirus, and reovirus.

Group	Virus	Strain
Enterovirus	Echo 7	Wallace
"	Echo 11	Gregory
"	Coxsackie B3	Nancy
"	Polio 1	Mahoney
Adenovirus	Adeno. 3	GB
"	Adeno. (not typed)	Tobao (patient)
Reovirus	Reovirus 1	Lang

C. Tissue cultures

For the early phases of this project, which are concerned with measuring viral titer differences resulting from variations in methods, it has been satisfactory and economical to use continuous passage lines propagated in this laboratory. For later phases of the project, one or more kinds of commercial primary cells will be purchased.

The BS-C-1 (BSC) line of African green monkey kidney and the HeLa line of human epithelioid cells were grown in M199 plus 10% inactivated fetal calf serum and antibiotics. The BSC line was prepared both as roller tube monolayer cultures and monolayers in 2 oz. prescription bottles for plaque tests. The HeLa line was used only for tube cultures.

D. Virus assays

It is our intention to quantitate the major virus groups in sewage in terms of infective viral units rather than TCD_{50}, comparing the plaque and MPN methods from the standpoint of practicability as well as accuracy. In our preliminary studies with stock virus strains we have included TCD_{50} titrations, because this laboratory utilizes the TCD_{50} method in routine diagnostic virology and we were interested in comparing it with the other two assay methods. Because viruses differ in plaquing efficiency under like circumstances, and from one set of culture conditions to another, studies are being made with stock strains to establish optimum procedures for those viruses recoverable from sewage and to determine relative plating efficiency compared to other assay systems. Selected viruses were titrated by one or more methods (TCD_{50}, MPN, PFU).

For tube tests, unless otherwise noted, the maintenance medium for BSC cultures was Eagle's MEM plus 2% inactivated agamma calf serum and antibiotics; for HeLa cultures, M199, 50%, Sherer's 50% plus 5% inactivated agamma calf serum and antibiotics.

For TCD_{50} titrations at least 4 tubes per dilution were inoculated. Endpoints were calculated by the method of Reed and Muench. MPN results were determined from a table prepared by Chang et al (3), using 5 tubes for each 4-fold dilution employed in the test. The initial dilution of the test was obtained through a series of 10-fold preliminary dilutions. For plaque tests, BSC monolayers in 2 oz. prescription bottles were used. Each bottle culture received 0.1 ml of inoculum. An adsoprtion period of 2 hours at 36C was followed by addition of 6 ml of agar overlay. Plaque overlay medium consisted of Earle's BSS, 0.4% $NaHCO_3$, 1% nonfat milk (restored dry product), 1:60,000 neutral red, and 1.5% Bacto-agar (Difco). For enteroviruses, 25 mM $MgCl_2$ was added (2). For reovirus tests, pancreatin was added, as recommended by Wallis et al (1). Buffered pancreatin tablets (Oxoid, Ltd.) were dissolved in distilled water in the proportion of 1 per 50 ml. water, the solution filtered through a 0.22 micron Millipore filter, and frozen as undiluted stock. It was incorporated in the overlay medium as a 1:30 or 1:60 final dilution.

Results and discussion

Comparison of assay methods Table 1 presents comparative titers obtained by different assay methods for echo. 7, echo. 11, reo. 1, and adeno. 3 prototypes. The TCD_{50} titers shown in parentheses were calculated from tests performed primarily for MPN results.

Dougherty (4) has stated that according to the Poisson distribution, other factors being comparable, the 50% effective dose would be expected to contain 0.69 infective particles per unit volume. Some of the comparative titers shown in Table 1 come very close to meeting this theoretical ratio, particularly the adeno.3 test, N409, and the echo. 11 test, N394, where TCD_{50} and MPN titers were both calculated from the same test results:

		Log_{10} titers per ml.		
Test	Virus	TCD_{50}	MPN	TCD_{50} X 0.69
N409	adeno. 3	8.40	8.22	8.24
N394	echo. 11	7.20	7.06	7.04

Technical considerations in plaque assays Inaccuracies introduced into plaque tests by coalescence of plaques, and the importance of permanently marking plaques during successive days of counting, as emphasized by Berg et al (5), became apparent in the use of 2 oz. prescription bottles. The plaques of polio 1 (Mahoney) started to coalesce when 30-40 were present per bottle.

By contrast, confluent plaques were not prominent in 2 oz. bottle cultures of reovirus 1 (Lang) until there were over 60 plaques per bottle, and not in echo. 7 (Wallace) cultures until the plaque number approached 100. With all 3 of these viruses there was a mixture of large, rapidly expanding and small, slowly expanding plaques, necessitating the use of inocula sufficiently dilute to limit the obscuring effect (5).

Plaquing of reovirus Wallis et al (1) reported that the low plaquing efficiency of reoviruses can be obviated by inclusion of the proteolytic enzyme, pancreatin, in the overlay medium. They further stated that the prototype reovirus 1 strain, Lang, fails to produce plaques without special treatment. Following their recommendations for the use of pancreatin, we obtained plaque titers for the Lang strain that compared favorably with tube tests (Table 1).

A plaque titration of reovirus 1 (Lang) was performed to compare PFU in the presence of 1:30 and 1:60 dilutions of pancreatin. Duplicate sets of five bottles were inoculated with 0.1 ml each of virus diluted 10^{-6} and 10^{-7}. The overlay medium for half the sets contained pancreatin at 1:30 dilution and the other half at 1:60 dilution. Nearly twice as many plaques were formed in the presence of the higher concentration of pancreatin, as shown below:

	Plaques per bottle Virus inoculum of 0.1 ml of 10^{-6}	
	Pan. 1:30	Pan. 1:60
Bottle #1	24	13
#2	22	12
#3	21	12
#4	21	9
#5	15	9
	Av. 20.6	Av. 11.0

Adenovirus assay Quantitation of adenoviruses presents a problem referable to their slow rate of growth. End point titrations performed in monolayer HeLa tube cultures demonstrated the necessity of observing these cultures for periods of 2 to 3 weeks. The progression of a typical adenovirus titration in HeLa tube cultures is shown below:

Virus	Strain	Log_{10} TCD_{50} per ml on day 3	7	11	14
Adeno. 3	GB	< 5.5	6.0	6.3	8.0

HeLa cells under agar do not survive for periods long enough to permit maximum development of adenovirus plaques. Assays for TCD_{50} or MPN have the advantage of permitting changes of maintenance medium.

We have not yet attempted plating adenoviruses, but intend to study the applicability of human fetal kidney cells, either primary or low passage level, for adenovirus plaque titrations. Monkey kidney cultures are insufficiently susceptible to adenoviruses.

E. Concentration of virus by adsorption to calcium and aluminum precipitates.

Preparation of precipitates for adsorption studies

Aluminum and calcium precipitates were prepared according to the methods of Wallis and Melnick (2), with the exception of final diluent volume.

Aluminum phosphate — Ten ml of 2M Na_2HPO_4 were added to 100 ml of 25mM $AlCl_3$.

Aluminum hydroxide — Three ml of 2M Na_2CO_3 were added to 100 ml of 25mM $AlCl_3$.

Calcium phosphate — Fifty ml of 0.02M $CaCl_2$ were mixed with 50 ml of 0.02M Na_2HPO_4.

In each case, the precipitates were centrifuged at 2000 rpm for 15 minutes and the supernatant fluid discarded. The sediment was suspended in 0.15M NaCl and centrifuged again. The supernatant was again discarded and the sediment resuspended in saline and autoclaved at 15 lbs. pressure for 15 minutes. After cooling, these suspensions were again centrifuged and the sediment of each was weighed.

$AlPO_4$: 0.6 ml of packed precipitate = 7.6 gms.
$Al(OH)_3$: 6.5 ml of packed precipitate = 9.4 gms.
$CaHPO_4$: 6.0 ml of packed precipitate = 1.15 gms.

Each sediment was resuspended in 50 ml of sterile saline and stored at 4C. Immediately before use, the suspension was mixed and the required volume centrifuged at 2000 rpm for 15 minutes, after which the sediment was resuspended to volume in pH 6.2 phosphate buffer (Sorensen's 0.1M prepared from Na_2HPO_4 and NaH_2PO_4) containing additional NaCl to give a final concentration of 0.15 molar Na ions.

Technique for adsorption of virus onto precipitates

Except for two experiments, in which one part of undiluted seed virus was added to 9 parts of precipitate suspension, the virus was initially diluted 1:10 or 1:100 in the buffer solution. The reason for this was to minimize any effect on viral adsorption of serum, tissue culture maintenance medium, or cellular products present in the seed virus preparation. One part of diluted virus was added to 9 parts of suspended precipitate, and one part of diluted virus was added to 9 parts of buffer solution for the virus control. These were held at room temperature for 30 minutes, with agitation each 5 minutes. The virus-precipitate mixture then was centrifuged for 5 minutes at 2000 rpm, the supernatant fluid decanted, and the sediment resuspended to volume in the pH 6.2

phosphate buffer. In some instances the sediment was washed once or twice in buffer before final centrifugation and resuspension to volume. Precipitate suspensions were assayed for adsorbed virus without any attempt to elute the virus. Virus controls always were assayed concurrently with the precipitate supernates and sediments.

Results and discussion

Table 2 presents results of experiments performed on adsorption of viruses to precipitates. On two occasions both supernate and resuspended sediment were assayed for virus; for the other tests, either supernate or sediment was assayed, but not both.

In confirmation of the work of Wallis and Melnick (2), these tests demonstrate the efficacy of calcium and aluminum precipitates to adsorb and concentrate viruses. Our results to date, however, do not show the adsorptive specificity of the three precipitates for different virus groups reported by these authors. We are attempting to determine what differences in technique may be responsible for this disparity.

Effect of serum on adsorption of virus to precipitate One experiment was performed to test the effect of serum on the adsorption of coxsackie B3 (Nancy) to $CaHPO_4$. Two tubes of precipitate suspended in phosphate buffer were prepared, one containing in addition 10% inactivated agamma calf serum. The virus was diluted 1:100 in buffer solution, and one part of diluted virus was added to 9 parts of each of the precipitate preparations, and to 9 parts of buffer for the virus control. After the usual adsorption period of 30 minutes at room temperature and centrifugation, the supernates were discarded. The sediments were resuspended to volume in buffer and then further diluted in Hanks' BSS to 10^{-7}. The virus control suspension was diluted in like manner. Three sets of 10 HeLa tube cultures were inoculated with 0.1 ml per tube of appropriate suspension at the 10^{-7} dilution. Results are shown as ratios of infected cultures to total cultures inoculated:

	Cultures infected/cultures inoculated
Virus control	6/10
Adsorption in absence of serum	8/10
Adsorption in presence of serum	1/10

In this test 10% agamma calf serum reduced adsorption of coxsackie B3 virus to $CaHPO_4$.

Effect of washing virus-precipitate complex Most of our experiments to date have not included washing of the virus-precipitate complex, and in only one instance was the complex assayed before and after being washed. Echo 11 virus, at a dilution of 10^{-3} in $AlPO_4$ suspension, was given a 30 minute adsorption period at room temperature, followed by centrifugation. The supernate was discarded, and after restoring the sediment to volume with buffer solution, a portion was removed for assay. The remaining portion was twice again centrifuged, supernates discarded, and sediment restored to volume in fresh buffer solution. The washed and unwashed suspensions were assayed in BSC tube cultures, 4 cultures per 10-fold dilution. TCD_{50} titers per ml. of suspension were:

Virus-precipitate complex not washed - $10^{4.50}$

Virus-precipitate complex washed 2X - $10^{4.67}$

No significant titer change resulted from the washing.

Recovery of enteroviruses from precipitates Our results pose the question of why enteroviruses recovered from precipitates titered higher than concurrent virus controls. Is the answer concerned with better dispersion of virus particles in the precipitate preparations? Or is the answer concerned with better virus survival during the course of the experiment in the presence of the precipitate? Future experiments will be directed toward finding an explanation.

F. Plans for next quarter

Plating of adenovirus for plaque tests will be attempted.

Further studies will be made of virus concentration by adsorption to precipitates.

Other concentration methods, such as adsorption of virus to Millipore filters, will be studied.

Sonication as an aid to dispersion of virus particles will be investigated in conjunction with concentration methods.

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Table 1. Virus assays by different methods: $\log_{10}$ titers per ml.

Virus[1]	Test No.	TC	TCD_{50}			MPN			PFU		
			Titer	Tubes per dil.	Dil. factor	Titer	Tubes per dil.	Dil. factor[2]	Titer	Bottles per dil.	Dil. factor
Echo 7	N388	BSC	(7.96)[3]	5	4	7.60	5	4			
"	R4	"							8.41	3	10
"	R7	"							8.03	5	10
Echo 11	N394	"	(7.20)	5	4	7.06	5	4			
"	N396	"	7.33	4	10						
"	N404	"	7.17	5	10						
Reo 1[4]	N391	"	8.0	4	10						
"	N405	"	(8.50)	5	4	8.42	5	4			
"	N410	"	8.38	5	10						
"	N411	"	8.32	5	10						
"	R12	"							8.31	5	10
"	R13	"							8.25	8	10
Adeno 3	N409	HELA	(8.40)	5	4	8.22	5	4			

1 For each virus, aliquots of a single seed lot were used.

2 For MPN tests, 10-fold dilutions of virus were made for initial dilutions not included in the test proper.

3 Parentheses indicate that TCD_{50} titers were calculated from tests designed for MPN determinations.

4 In each instance, reovirus was inoculated into drained cultures, the inoculum removed after a 2 hr. adsorption period at 36C., and the cell layer washed once with Hanks' BSS before addition of maintenance medium or overlay. Maintenance medium for N410 was Eagle's, agamma calf serum 2%. For N391, 405, and 411 the maintenance medium was Eagle's, nonfat milk 2%. N405 also contained pancreatin to final dilution of 1:30, replaced on 4th day of test with Eagle's, agamma calf 2%.

Table 2. Adsorption of viruses to aluminum and calcium precipitates: $\log_{10}$ titers per ml.

Virus	Strain	Seed lot no.	Control[1]	$AlPO_4$				$Al(OH)_3$				$CaHPO_4$			
				SN[2]	%	SED[3]	%	SN	%	SED	%	SN	%	SED	%
Echo 7	Wallace	TC3444	6.50 TCD_{50}	5.75 TCD_{50}	18			4.25 TCD_{50}	0.56			3.50 TCD_{50}	0.10		
Echo 7	Wallace	N333	6.03 PFU											6.07 PFU	>100
Echo 11	Gregory	TC2943	4.33 TCD_{50}			4.59 TCD_{50}	>100								
Echo 11	Gregory	"	4.17 TCD_{50}	3.62 TCD_{50}	29	4.38 TCD_{50}	>100								
Cox. B3	Nancy	TC2053	6.0 TCD_{50}							6.33 TCD_{50}	>100			6.33 TCD_{50}	>100
Reo 1	Lang	TC2932	5.25 PFU					4.96 PFU	52	5.07 PFU	67				
Adeno	Tubac	TC1614	7.0 TCD_{50}							7.0 TCD_{50}	100				

1 Quantity of virus applied per ml of precipitate suspension, determined by concurrent assay of virus control suspension.

2 Supernate.

3 Sediment resuspended to original volume.

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